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Biochimica et Biophysica Acta 1453 (1999) 185–192

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Maroteaux–Lamy syndrome: five novel mutations and their structural localization

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Received 23 October 1998; accepted 29 October 1998

Abstract

Maroteaux–Lamy syndrome (mucopolysaccharidosis type VI, MPS VI) is an autosomal recessive disorder due to the deficiency of the lysosomal enzyme *N*-acetylgalactosamine-4-sulfatase (arylsulfatase B, ASB). Mutation analysis in Maroteaux–Lamy syndrome resulted in the identification of approximately 40 molecular defects underlying a great genetic heterogeneity. Here we report five novel mutations in Italian subjects: S65F, P116H, R315Q, Q503X, P531R; each defect was confirmed by restriction enzyme or amplification refractory mutation system (ARMS) analysis. We also performed a three-dimensional (3-D) structure analysis of the alterations identified by us, and of an additional 22 point mutations reported by other groups, in an attempt to draw helpful information about their possible effects on protein conformation.

Keywords: Mucopolysaccharidosis type VI; Mutation analysis

1. Introduction

Mucopolysaccharidosis type VI (MPS VI), Maroteaux–Lamy syndrome, is a glycosaminoglycan storage disorder caused by the deficiency of the lysosomal enzyme arylsulfatase B (ASB, *N*-acetylgalactosamine-4-sulfatase, EC 3.1.6.12) that is required for degradation of glycosaminoglycans dermatan sulfate and chondroitin sulfate [1]. A deficiency of this enzyme causes urinary excretion and intralysosomal accumulation of large amounts of partially degraded dermatan sulfate leading to variable clinical symptoms: according to the age of onset and progression of symptoms, three forms of the disease can be differentiated (infantile or severe, juvenile or intermediate, and adult form or mild).

Arylsulfatase B and arylsulfatase A are the only sulfatases for which the crystal structure has been solved and refined at 2.5–2.1 Å resolution [2,3]. As for all sulfatases, ASB was reported by sequence alignment [4,5] to contain a CTPSR 'common sulfatase motif' where the Cys 91 residue plays an important role in enzyme function. In fact this residue has been shown to undergo a post-translational modifi-

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0925-4439/99/$ – see front matter
PII: S0925-4439(98)00099-4

cation to 2-amino-3-oxopropionic acid [6]; the subsequent reaction scheme(s) for sulfate ester cleavage has recently been proposed [2,7]. The conserved residues that line the ASB active sites are Asp 53, Asp 54, Cys 91, Pro 93, Ser 94, Arg 95, Lys 145, His 147, His 242, Asp 300 and Lys 318; moreover a metal ion (Ca^{2+}) has been proposed to be involved in the active site [2].

Isolation of the ASB cDNA [8,9] and characterization of the exon/intron structure of the ASB gene [10] allowed analysis of the molecular basis of this disease, leading to the identification of several ASB gene mutant alleles [11–19]. The biochemical effects of the identified mutations on the ASB polypeptides were studied by in vitro expression for few defects [11,14,15,17].

In this study we report the identification of five novel mutations in Italian patients with varying clinical severities and their structural localization using a 3-D structural analysis.

2. Materials and methods

2.1. Patients

Fibroblasts of patients Mo., Br. and La. were obtained from Prof. Gatti of the Gaslini Institute, Genova; lymphoblasts of patient Gu. were obtained from Prof. Fois of the Pediatric Clinic, University of Siena, and lymphoblasts of patient Ri. were from Prof. Andria of the Department of Pediatrics, University of Naples. Arylsulfatase B deficiency was assessed by demonstration of severely decreased enzyme activity in patients' cells. Classification of subjects in severe, intermediate or mild forms (Table 1) was from the above mentioned clinicians who followed the criteria of Neufeld and Muenzer [1]. In this classification, the severe form is characterized by very early onset and severe progression of the symptoms: facial dysmorphism, skeletal abnormalities, compression of the spinal cord, corneal clouding, hepatosplenomegaly, mental retardation (patient Gu. was diagnosed at age 14 months); the mild form is characterized by the very late onset and absence of mental retardation (patient Ri. was diagnosed at 7 years of age), while the intermediate form is in the middle of such spectrum of phenotypes.

2.2. Genomic DNA extraction, PCR experiments and SSCP analysis

Genomic DNA was obtained from patients' fibroblasts and from normal controls as described [20]. For both cases, mutation screening was performed using single-strand conformation polymorphism (SSCP) analysis as previously reported [19]. Polymerase chain reaction (PCR) conditions for amplification of patients DNA were according to Isbrandt et al. [15], except for exon 5, where amplification was obtained using the following primers: ABE1 (5′-TCAGATAACGGAGGGCAGA-3′) and ABE2 (5′-GGTACCTGATGGTTTTCCA-3′).

2.3. DNA sequencing

PCR-amplified DNA fragments were sequenced directly, without subcloning, by fluorescent dye terminator cycle sequencing, and analysis was performed on an automated DNA-Sequencer (Applied Biosystems).

2.4. Restriction enzyme and ARMS analysis

After complete sequencing of the PCR products, restriction enzyme analysis or amplification refractory mutation system (ARMS) analysis [21] were done to confirm the authenticities of the novel mutations. In parallel to patients' samples, DNA from

Table 1
Mutations found in the ARSB gene of Italian MPS VI patients

Patients	Phenotype	Exon	Mutation	Polymorphism	
				exon	Amino acid change
Mo.	Intermediate	1	S65F	2	I114I (sil)
				2	L124L(sil)
				5	G324G (sil)
				5	V358M
Gu. (+)	Severe	2	P116H		
Br.	Intermediate	5	R315Q		
La.	Intermediate	8	Q503X	2	L124L (sil)
				5	V358M
Ri. (+)	Mild	8	P531R		

(+): Consanguinity.

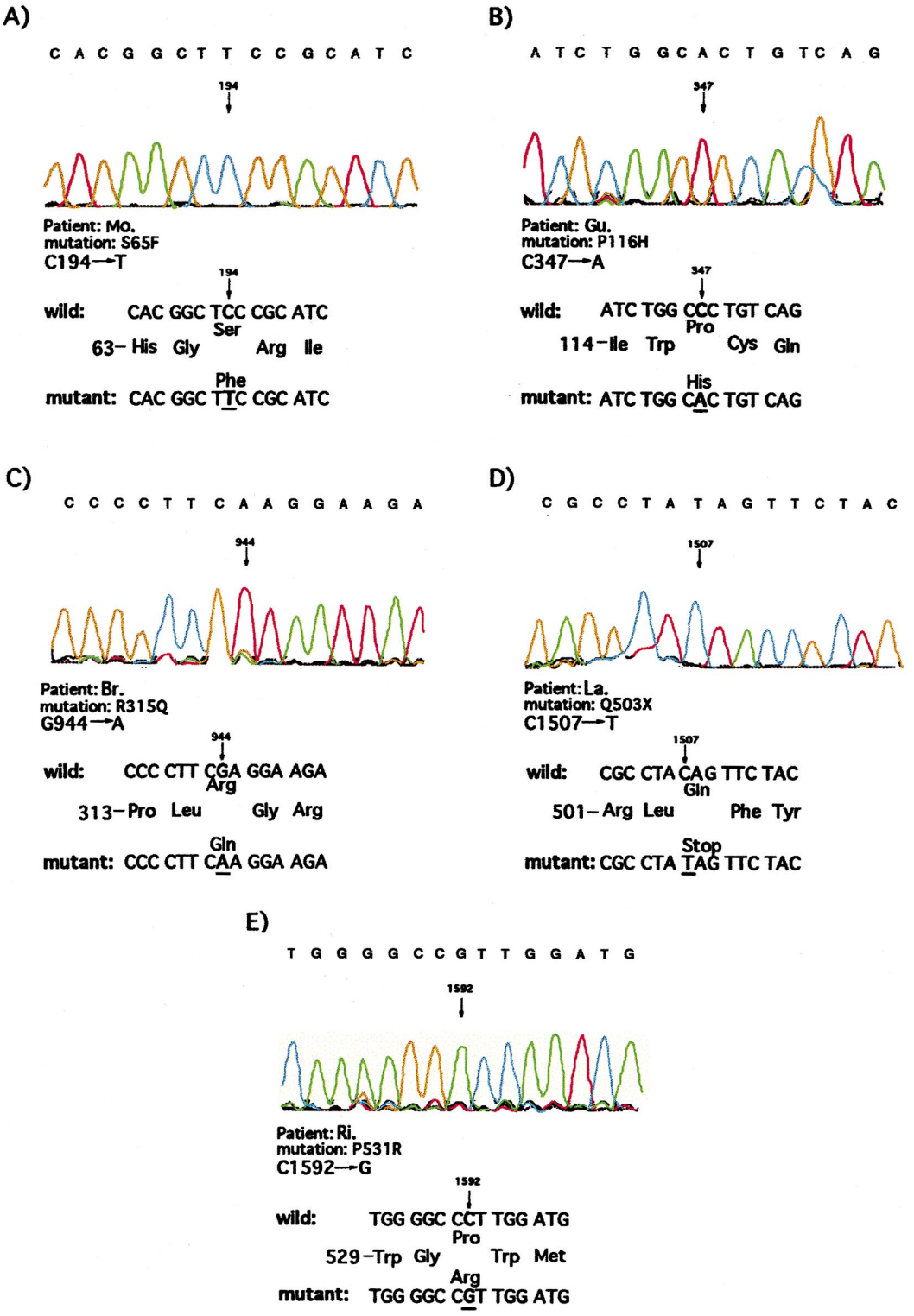

Fig. 1. Identification of point mutations in ASB gene exons of five MPS VI patients. Sequencing diagrams covering positions of nucleotide alterations are shown. The nucleotide sequence is given above the graphs; nucleotide alterations in comparison with the wild type ASB cDNA are arrowed. Patient initials, mutation and nucleotide alteration are listed.

Fig. 2. ARMS and restriction analysis of the identified mutations. (A,D) ARMS test for S65F and Q503X mutations. M is the molecular marker 100 bp ladder. Lanes 1 and 2, normal control; lanes 3 and 4, patients. Lanes 1 and 3, the genomic DNA was amplified by using the normal primer; lanes 2 and 4, the genomic DNA was amplified by using the mutant primer. All the reactions included as common primer AB92 for S65F mutation, and AB68 for Q503X alteration; SAG1/SAG2 primers were used to amplify the internal control fragment (see Section 2). On the bottom: a schematic representation of the ASB gene showing the localization of the primers used in the ARMS analysis. (B,C,E) Restriction enzyme analyses for P116H, R315Q, P531R mutations. PCR amplified genomic DNA fragments were digested as described in Section 2 and the products were separated on a 2% agarose gel. M, 100 bp DNA ladder. Lane 1, normal control; lane 2, patient; lane 3, undigested samples.

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10 normal subjects was also examined. Restriction enzyme digestion was performed on mutations P116H, R315Q and P531R. Enzymes were purchased from New England Biolabs (Beverly, MA). Genomic DNA samples were amplified by PCR and purified by Agarose Gel DNA Extraction kit (Boehringer Mannheim) or Qiagen gel extraction kit (Diagen) as described by the manufacturer. After purification, digestion was performed as recommended by the manufacturer.

ARMS analysis was performed on mutations S65F and Q503X using allele specific primers. For S65F mutation the following primers were used: mS65F (mutant primer containing the mutant base at 3′ and a additional deliberate mismatch G/C three bases from the 3′ end: 5′-GCGCGTCCAGGTGCGGCGTGCGGATGCCGA-3′) or nS65F (normal primer containing only the additional mismatch: 5′-GCGCGTCCAGGTGCGGCGTGCGGATGCCGG-3′); the common primer was AB92, as named by Isbrandt et al. [15]. For Q503X mutation, primers were the following: mQ503X (containing the mutant base and a T/A mismatch three bases from the 3′ end: 5′-ACATCGTCACAAAGCTCCTGTCCCGCCAAT-3′) or nQ503X (containing only the T/A mismatch: 5′-ACATCGTCACAAAGCTCCTGTCCCGCCAAC-3′); the common primer was AB68 [15]. All the reactions contained primers SAG1/SAG2 amplifying a fragment of 392 bp corresponding to exon 7 of heparin *N*-sulfamidase gene as internal control [22].

2.5. Graphical analyses

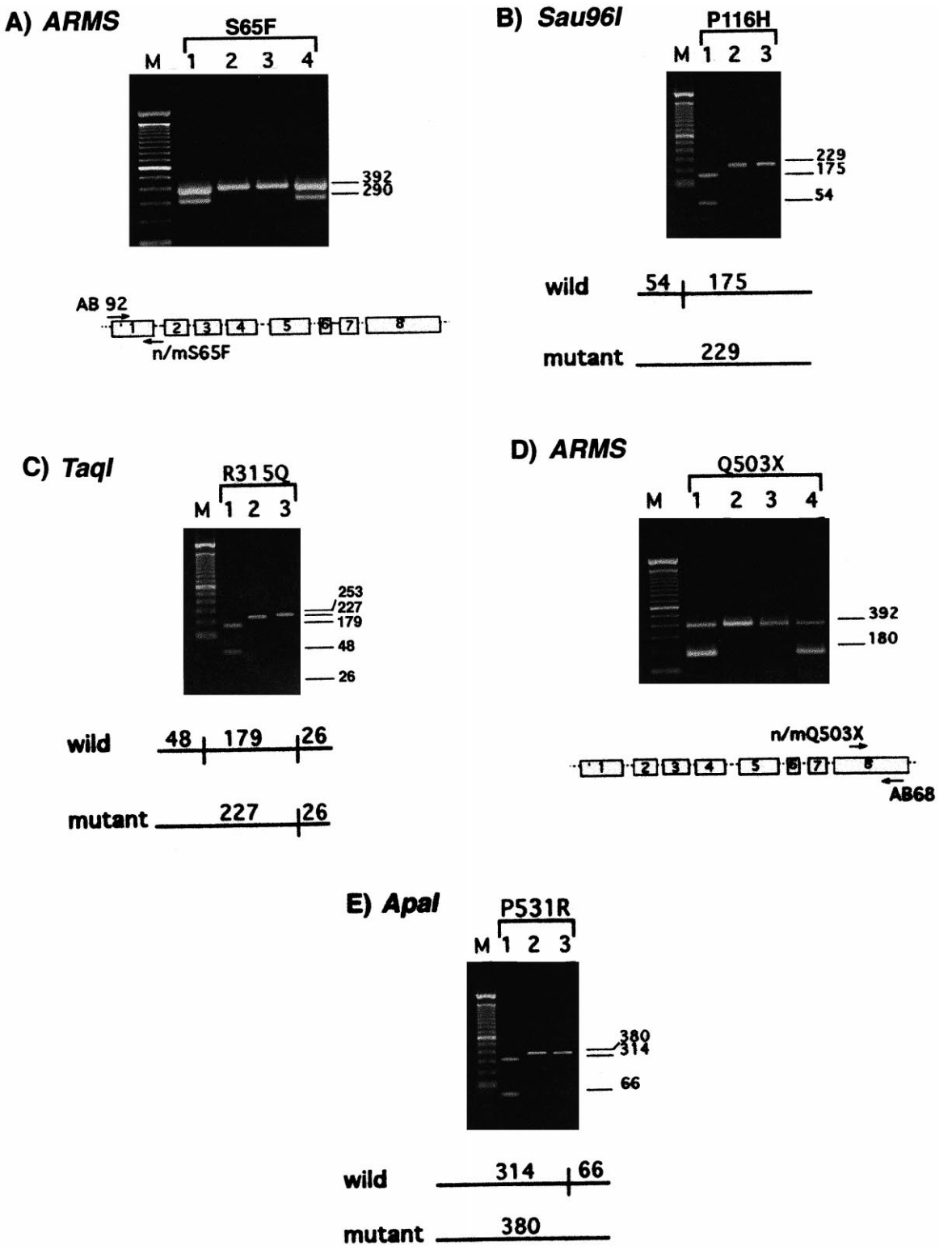
All graphical analyses were run on a Silicon Graphics Indigo2 workstation, using the package INSIGHT II (Biosym Technologies). The co-ordinates for the human lysosomal sulfatase (*N*-acetylgalactosamine-4-sulfatase) X-ray structure at 2.5 Å resolution [2] were obtained from Protein Data Bank (a) (1FSU) (Brookhaven National Laboratory, Upton, NY).

3. Results

To identify possible exonic mutations in the ASB gene of five Italian patients, the entire open reading frame was PCR-amplified on eight DNA fragments, each covering an exon and its flanking regions. The obtained exons were screened by SSCP analysis to identify fragments with anomalous patterns [19]. DNA sequence analysis was performed by direct fluorescent dye-terminator cycle sequencing as described in Section 2. In all five cases the alterations were shown to be homoallelic, although only two patients were the outcome of a consanguineous marriage (Table 1).

In patient Mo., SSCP alterations were found in exons 1, 2 and 5 (Table 1). DNA sequencing showed a homoallelic C to T transition at position 194, which resulted in a Ser to Phe change at residue 65 (S65F; Fig. 1A). In addition to the disease-causing mutation, in this patient, sequence analysis showed the existence of several polymorphisms concerning exon 2 (I114I and L124L) and exon 5 (G324G and V358M). Of these, V358M was previously reported to be a polymorphism [9,11]. In exon 2, the patient was found to be homozygous for the silent T to C transitions at nucleotides 342 and 370, in the third base of a Ile codon at amino acid 114 and in the first base of a Leu codon at amino acid 124, respectively. In exon 5, a homoallelic silent A to G transition was found at nucleotide 972 in the third base of a Gly codon, at amino acid 324 (data not shown).

In patient Gu., the sequence of exon 2 revealed a single base substitution, a C to A transversion at nucleotide 347, which resulted in a Pro to His re-


A) ARMS
S65F
M 1 2 3 4
392
290
AB 92
1 2 3 4 5 6 7 8
n/mS65F
B) Sau96I
P116H
M 1 2 3
229
175
54
wild
54 175
mutant
229
C) TaqI
R315Q
M 1 2 3
253
227
179
48
26
wild
48 179 26
mutant
227 26
D) ARMS
Q503X
M 1 2 3 4
392
180
n/mQ503X
1 2 3 4 5 6 7 8
AB68
E) ApaI
P531R
M 1 2 3
380
314
66
wild
314 66
mutant
380

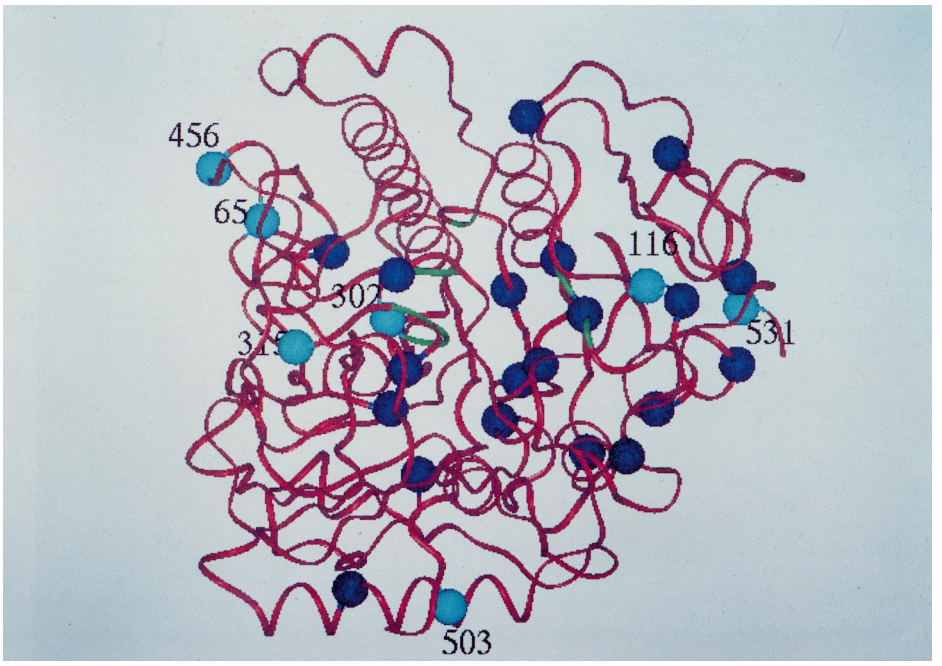


Fig. 3. Molecular model of *N*-acetylgalactosamine-4-sulfatase. The model shows the seven alterations found in Italian patients (turquoise) and the other mutations analysed (blue). The active site is shown in green.

placement at residue 116 (P116H; Fig. 1B). A G to A transition at nucleotide 944 of the exon 5 was found in patient Br., which resulted in a Arg to Gln change at residue 315 (R315Q; Fig. 1C). In patient La., a C to T transition at nucleotide 1507 created a nonsense mutation that changed CAG (Gln) to TAG (stop) at codon 503 in exon 8 (Q503X; Fig. 1D). The patient was also found to be homozygous for the polymorphisms L124L and V358M (Table 1). Finally, for patient Ri., sequence analysis revealed a C to G transversion at nucleotide 1592 which caused a Pro to Arg substitution at residue 531 in exon 8 (P531R; Fig. 1E).

Sequence analysis revealed that none of the five mutant alleles identified here was found in 50 normal ASB alleles from unrelated healthy subjects; moreover, the Q503X results in a stop codon. These results suggest that the identified molecular alterations are mutations causing functional defects in the encoded ASB polypeptides rather than polymorphisms. The new mutations were confirmed by restriction enzyme or ARMS analyses, on PCR-amplified genomic DNA (Fig. 2). As shown in Fig. 2A, ARMS analysis performed using appropriate primers described in Section 2 confirmed that patient Mo. was homoallelic for the S65F mutation, since his exon 1 DNA was amplified only with mutant primer mS65F. ARMS test confirmed also that patient La. had a homozygous condition for Q503X mutation (Fig. 2D). The P116H mutation caused, in the homoallelic patient Gu., the disappearance of a restriction site for *Sau 96 I* enzyme that in wild-type DNA cleaves a 229 bp PCR product to 175 and 54 bp (Fig. 2B). R315Q alteration in the homozygous subject Br. abolishes a *Taq*I site whitin exon 5, leading, after digestion, to 227 and 26 bp fragments instead of 179, 48 and 26 bp found in wild-type DNA (Fig. 2C). Finally, P531R mutation in the homoallelic patient Ri. destroys the *Apa*I resulting in a 380 bp fragment, replacing the normal situation of 314 and 66 bp fragments (Fig. 2E).

All these five mutations, together with the other two previously described by us (G302R, Q456X) and with 22 point mutations reported by different laboratories (L72Q, T92M, R95Q, L98P, C117R, G137V, G144R, W146X, R152W, R160Q, R160X, C192R, Y210C, L236P, R315X, S320R, L321P, H393P, C405Y, L498P, Y513X, C521Y), were graphically analysed as described in Section 2. Results of this analysis are shown in Fig. 3.

4. Discussion

We report here five novel mutations in arylsulfatase B gene: S65F, R315Q and Q503X associated to intermediate phenotypes, P116H found in a severely affected patient and P531R associated with a mild subject. We also report the identification of three silent mutations in exons 2 and 5 (I114I, L124L, G324G), but at this time we are not able to define if these polymorphisms could have some effect on clinical phenotypes, as suggested for MPS I disease [23]. It should be noted that all the five mutations were found in homozygosity although only for two subjects (Gu. and Ri., Table 1) consanguinity was proven. For the other three patients, since it is unusual that a subject with a rare disease, such as MPS VI occurring in less than 1:100 000 births, would be homoallelic for a mutation, unless the mutant allele was common or the patient was a product of a consanguineous mating, this latter explanation is presumed possible.

None of the five alterations reported here involve amino acids that are conserved between the eukaryotic sulfatases aligned so far [4,5,8]. Nonetheless we hypothesize that these amino acids may play a role in enzyme's structure and/or function. The missense S65F mutation concerns an amino acid which may be critical for enzyme function, since position 65 is located in close proximity to the 53-Asp–Asp-54 dipeptide involved in the active site. The substitution of a polar amino acid (Ser) with a hydrophobic one (Phe) could alter the contacts between the Asp residue with the metal ion Ca^{2+} [2]. For P116H mutation, since it was reported that enzyme–substrate contacts are made also by Pro116 [2], one can speculate that introduction of a positive charge in this position could interfere with contacts formation. Likewise, Arg315 substitution with Gln residue leads to the loss of a positive charge in an important region involved both in substrate binding and metal ion coordination. Accordingly, all these mutations lead to severe or intermediate phenotypes.

The nonsense mutation Q503X results in the translation of a putative mutant enzyme lacking the last 30 amino acids. Because this truncation predicts the loss a disulfide bridge, it is possible that this alteration may cause a major change of the overall structure of the enzyme resulting in a intermediate phenotype. Finally, mutation P531R concerns the third amino acid from the C-terminus, thus it is unlikely that it would result in a non-functional protein. Most likely, since the C-terminal portion of ASB protein has been shown to interact with a peculiar β-hairpin structure presumably involved in mannose-6-phosphate acquisition [2], an alteration in this region (i.e., P531R) could prevent the normal targeting of the mutant protein. On the other hand, a putative minimal amount of this altered protein could justify the mild phenotype of patient Ri.

Graphical analysis of seven molecular defects identified by our group (Fig. 3, turquoise) ([19]; this report) showed that no alteration occurred in the active site. When this analysis was extended to 22 point mutations (Fig. 3, blue) identified by others [11,12,15–17,24], only R95Q was shown to occur in the active site, causing the loss of an H-bond between the $N^{\varepsilon}H$ in Arg side chain and the hemiacetal group. In conclusion, most of the mutations on ASB gene seem to lead to structural impairment of the protein folding, and, consequently, of the stability of the mature form of the enzyme.

Acknowledgements

Supported by 'Contributo cofinanziamento Programmi di Ricerca di Interesse Nazionale Es. Fin. 1997' and by Telethon-Italy (Project no. E 812).

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